

Stabilin-1 expression defines a subset of macrophages that mediate tissue homeostasis and prevent fibrosis in chronic liver injury

Rantakari, Pia; Patten, Daniel; Valtonen, Joonas; Karikoski, Marika; Gerke, Heidi; Dawes, Harriet; Laurila, Juha; Ohlmeier, Steffan; Elimä, Kati; Hubscher, Stefan; Weston, Christopher; Jalkanen, Sirpa; Adams, David; Salmi, Marko; Shetty, Shishir

DOI:

[10.1073/pnas.1604780113](https://doi.org/10.1073/pnas.1604780113)

Document Version

Peer reviewed version

Citation for published version (Harvard):

Rantakari, P, Patten, D, Valtonen, J, Karikoski, M, Gerke, H, Dawes, H, Laurila, J, Ohlmeier, S, Elimä, K, Hubscher, S, Weston, C, Jalkanen, S, Adams, D, Salmi, M & Shetty, S 2016, 'Stabilin-1 expression defines a subset of macrophages that mediate tissue homeostasis and prevent fibrosis in chronic liver injury', *National Academy of Sciences. Proceedings*, vol. 113, no. 33, pp. 9298–9303. <https://doi.org/10.1073/pnas.1604780113>

[Link to publication on Research at Birmingham portal](#)

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Download date: 05. May. 2023

Biological Sciences

Stabilin-1 expression defines a subset of macrophages which mediate tissue homeostasis and prevent fibrosis in chronic liver injury

Pia Rantakari^{1*}, Daniel A. Patten^{2*}, Joonas Valtonen¹, Marika Karikoski¹, Heidi Gerke¹, Harriet Dawes², Juha Laurila¹, Steffen Ohlmeier³, Kati Elimä¹, Stefan G. Hubscher⁴, Christopher J. Weston², Sirpa Jalkanen¹, David H. Adams², Marko Salmi¹, Shishir Shetty²

1. MediCity Research Laboratory, and Department of Medical Microbiology and Immunology, University of Turku, Turku, Finland.
2. National Institute for Health Research (NIHR) Biomedical Research Unit and Centre for Liver Research, Medical School, University of Birmingham, Birmingham, United Kingdom.
3. Proteomics Core Facility, Biocenter Oulu, Faculty of Biochemistry and Molecular Medicine, University of Oulu, Oulu, Finland
4. School of Cancer Sciences, University of Birmingham, Birmingham, United Kingdom

Corresponding Author- Shishir Shetty

Corresponding Address- Centre for Liver Research, 5th Floor, IBR, Medical School, University of Birmingham, UK, B15 2TT.

Phone: 44.0.121.415.8700

Fax: 44.0.121.415.8701

E-mail. s.shetty@bham.ac.uk

* These authors contributed equally to this work

34 **Abstract**

35 Macrophages are key regulators of fibrosis development and resolution. Elucidating the
36 mechanisms by which they mediate this process is crucial for establishing their therapeutic
37 potential. Here, we use experimental models of liver fibrosis to show that deficiency of the
38 scavenger receptor, stabilin-1, exacerbates fibrosis and delays resolution during the
39 recovery phase. We detected a subset of stabilin-1⁺ macrophages which were induced at
40 sites of cellular injury close to the hepatic scar in mouse models of liver fibrosis and in
41 human liver disease. Stabilin-1 deficiency abrogated malondialdehyde-LDL (MDA-LDL)
42 uptake by hepatic macrophages and was associated with excess collagen III deposition.
43 Mechanistically, the lack of stabilin-1 led to elevated intrahepatic levels of the pro-fibrogenic
44 chemokine CCL3 and an increase in GFAP⁺ fibrogenic cells. Stabilin-1^{-/-} macrophages
45 demonstrated a pro-inflammatory phenotype during liver injury and the normal induction of
46 Ly6C^{lo} monocytes during resolution was absent in stabilin-1 knockouts leading to
47 persistence of fibrosis. Human stabilin-1⁺ monocytes efficiently internalised MDA-LDL and
48 this suppressed their ability to secrete CCL3 suggesting that loss of stabilin-1 removes a
49 brake to CCL3 secretion. In support of this, studies with cell lineage specific knockouts
50 revealed that stabilin-1 expression in myeloid cells is required for the induction of this novel
51 subset of macrophages and that increased fibrosis occurs in their absence. This study
52 demonstrates a new regulatory pathway in fibrogenesis in which a macrophage scavenger
53 receptor protects against organ fibrosis by removing fibrogenic products of lipid
54 peroxidation. Thus stabilin-1⁺ macrophages shape the tissue microenvironment during liver
55 injury and healing.

56

57

58

59

60 **Significance Statement**

61 Organ fibrosis is a major cause of global morbidity and mortality. It is driven by chronic
62 inflammation and associated oxidative stress with depletion of cellular antioxidant defenses.
63 We demonstrate a novel mechanism in which the evolutionarily conserved receptor stabilin-
64 1 on tissue-infiltrating macrophages provides a second-line defense to prevent tissue
65 damage from oxidative stress. Stabilin-1⁺ monocytes take up malondialdehyde-LDL (MDA-
66 LDL), a major product of oxidative lipid peroxidation, to form ceroid-laden macrophages.
67 Through the uptake of MDA-LDL, stabilin-1 suppresses production of the pro-fibrogenic
68 chemokine CCL3 and prevents excessive collagen deposition in experimental models of
69 liver fibrosis. We propose that macrophage stabilin-1 is a critical defense against oxidative
70 tissue damage and thereby maintains tissue homeostasis.

71

72 /body

73 **Introduction**

74 Liver fibrosis is driven by extracellular matrix (ECM) deposition by activated myofibroblasts,
75 the majority of which arise from the hepatic stellate cell (HSC) (1). The accumulation of an
76 ECM rich in fibrillar collagens I and III, causes architectural distortion which leads to organ
77 dysfunction and portal hypertension. Consequently, identifying pathways which regulate the
78 deposition and resolution of fibrillar collagen is vital to developing new treatments for
79 chronic liver disease. Products of oxidative stress, such as oxidised-low density lipoproteins
80 (oxLDLs), can directly activate HSC to an ECM-producing state thereby driving fibrogenesis
81 (2, 3). In addition to HSCs, other non-parenchymal cells, contribute to the regulation of liver
82 fibrosis. Hepatic sinusoidal endothelial cells (HSEC), which lie in close proximity to HSCs,

83 play an important role in maintaining HSC quiescence (4), and macrophages play a critical
84 role in fibrosis progression, tissue remodelling and resolution (5).
85 Stabilin-1, also known as CLEVER-1 (gene name *Stab1*), is a highly conserved
86 transmembrane glycoprotein that is expressed by sinusoidal endothelium, and sub-
87 populations of macrophages (6). Previous studies have demonstrated multiple stabilin-1
88 ligands including oxLDL (7), the ECM glycoprotein osteonectin (8) and placental lactogen
89 (9) suggesting that stabilin-1 functions as a homeostatic scavenger receptor. We have
90 previously reported stabilin-1 expression by HSEC, but not by resident liver macrophages
91 (known as Kupffer cells), in normal human liver (10). Our observation of increased
92 expression in a range of chronic inflammatory liver diseases led us to study whether
93 stabilin-1 contributes to the progression of chronic liver disease and fibrogenesis.

94

95 **Results**

96 **Stabilin-1 deficiency exacerbates liver fibrosis**

97 To investigate the functional role of stabilin-1 in liver injury and fibrosis we used two mouse
98 models. In carbon tetrachloride (CCl₄)-induced chronic liver injury a fibrogenic phase is
99 followed by a spontaneous resolution phase (11) and in mice fed a methionine choline
100 deficient (MCD) diet hepatic steatosis is followed by inflammation and early fibrosis (12).

101 Consistent with previous reports in stabilin-1^{-/-} mice, sirius red positive fibres consistent with
102 increased fibrogenesis were seen within the liver parenchyma which was otherwise normal
103 (Fig.1A,B) (13). Following CCl₄ administration, stabilin-1^{-/-} mice developed a marked
104 increase in hepatic scar formation compared with WT mice with features of bridging fibrosis
105 (Fig.1A,B) and increased sirius red staining (SI Appendix Fig.S1A). Greater activation of
106 fibrogenic pathways in stabilin-1^{-/-} mice was confirmed by significantly increased
107 accumulation of collagen I and III (Fig.1C,D, SI Appendix Fig.S1B,C).). In order to study

108 the number and activation of fibrogenic cells at baseline and injury we compared the
109 expression of glial fibrillary acidic protein (GFAP) as a constitutive marker of stellate cells to
110 alpha smooth muscle actin (α SMA) and matrix metalloproteinase-2 (MMP-2) as markers of
111 activated myofibroblasts (14, 15). At baseline and throughout injury and resolution we
112 detected increased GFAP expression in livers of stabilin-1^{-/-} mice (Fig.1E-H). In contrast
113 α SMA and MMP-2 expression was significantly higher only during injury (Fig.1E-H, SI
114 Appendix Fig.S1D,E) compared to WT mice.

115 The role of stabilin-1 in the resolution of fibrosis was studied in mice during recovery after
116 CCl₄ injury. After four weeks of recovery, we noted persistent bridging fibrosis and
117 increased collagen I and III transcription in stabilin-1^{-/-} mice when compared to WT mice
118 (Fig.1I,J). Hydroxyproline quantification demonstrated an increase in collagen content in
119 stabilin-1^{-/-} mice at baseline comparable to that seen in WT mice after CCl₄ administration
120 (Fig.1K). After four weeks of resolution, the hydroxyproline had reduced in WT mice but
121 increased further in stabilin-1^{-/-} mice (Fig.1K). We also noted that stabilin-1^{-/-} mice
122 demonstrated significantly increased ALT levels during liver injury (Fig.1L). To assess if
123 elevated ALT levels were due to increased hepatocyte death or inefficient scavenging we
124 co-stained for ALT and F4/80 and assessed markers of cell death. We found increased co-
125 expression of ALT and F4/80 in WT mice compared to stabilin-1^{-/-} mice, but no significant
126 difference in levels of apoptosis and autophagy (SI Appendix Fig. S2 A-I). Histological
127 assessment demonstrated minimal necrosis with no difference between WT and stabilin-1^{-/-}
128 mice at baseline or after 8 weeks of carbon tetrachloride (SI Appendix Fig. S2 J). These
129 findings suggest inefficient scavenging in stabilin-1^{-/-} mice rather than increased cellular
130 death.

131 We also detected histologically more severe scarring in stabilin-1^{-/-} MCD diet fed mice and
132 significantly increased hydroxyproline levels compared to WT MCD diet fed mice, as well as,

133 higher, but not statistically significant, ALT levels (SI Appendix Fig.S3 A-E). Collectively
134 these data show that *in vivo* there is an increased baseline fibrogenic response in stabilin-1⁻
135 mice, which is due to increased numbers of fibrogenic cells. Fibrosis and scarring are
136 enhanced upon injury, and the resolution phase is impaired in the absence of stabilin-1.

137 **Injury-dependent induction of stabilin-1 in hepatic macrophages**

138 We proceeded to study the cellular expression of stabilin-1 in normal and injured murine
139 livers. In uninjured WT mice, stabilin-1 was restricted to CD31-positive sinusoidal
140 endothelial cells and was absent from F4/80-positive Kupffer cells (SI Appendix Fig. S4A,B).
141 In response to liver injury from either CCl₄ or MCD diet we detected a subset of stabilin-1⁺
142 F4/80⁺ intrahepatic macrophages (SI Appendix Fig.S4C, S5A). Using αSMA as a cell
143 lineage marker, we clearly demonstrated that myofibroblasts, which play a central role in
144 liver fibrosis, do not express stabilin-1 during liver injury (SI appendix Fig. S4D, S5B).
145 Analysis of chronic human liver disease demonstrated that CD31-positive sinusoidal
146 endothelial cells expressed stabilin-1 whereas CD68-positive Kupffer cells within the
147 sinusoids did not (SI Appendix Fig. S4E,F). In contrast, stabilin-1⁺ macrophages were
148 readily detected within the fibrous septa associated with the hepatic scar (SI Appendix
149 Fig.S4G). Stabilin-1 was not detected on αSMA-positive myofibroblasts (SI Appendix Fig.
150 S4H). These studies demonstrate for the first time an intrahepatic subpopulation of stabilin-
151 1⁺ macrophages in liver injury.

152

153 **Stabilin-1 deficiency leads to a reduction of ceroid macrophages in liver injury.**

154 The presence of a population of stabilin-1⁺ macrophages in liver injury and previous
155 findings that macrophages are critical to the development of fibrosis (16), led us to
156 investigate whether an alteration in macrophage distribution or function contributes to the
157 increased fibrosis in stabilin-1^{-/-} mice. Intrahepatic macrophage distribution and peripheral

158 blood monocyte numbers were comparable between uninjured WT and stabilin-1^{-/-} mice
159 (Fig.2A,B, SI Appendix Fig S9A). After CCl₄ injury, WT mice demonstrated prominent
160 aggregates of macrophages, which were not seen in the stabilin-1^{-/-} animals (Fig.2A-C).
161 These macrophage aggregates resembled ceroid-laden macrophages. Ceroid-laden
162 macrophages contain lipid-like pigment deposits and have been previously described in
163 experimental liver fibrosis but their pathological significance is unknown (17, 18).
164 We hypothesized that stabilin-1 deficiency was associated with a reduction in ceroid-laden
165 macrophage formation during liver injury and fibrosis. To test this hypothesis, we used
166 periodic acid-schiff diastase (PAS-D) staining to detect cytoplasmic ceroid accumulation.
167 This confirmed the presence of large ceroid macrophages in WT mice (Fig.2D) that were
168 largely absent in stabilin-1^{-/-} animals (Fig.2D). Interestingly, PAS-D also stains the fibrous
169 scar and in the stabilin-1^{-/-} mice the absence of ceroid-laden macrophages was associated
170 with prominent fibrosis (Fig.2D).
171 MDA is the most abundant aldehyde produced by lipid peroxidation and is highly reactive
172 leading to the formation of MDA-modified LDL. This product of oxidative stress is taken up
173 by macrophages and contributes to cytoplasmic ceroid accumulation (19). In WT mice,
174 prominent MDA-positive cells were clearly visible (Fig.2E), whereas minimal staining was
175 detected in stabilin-1^{-/-} mice (Fig.2E,F).
176 A further well established property of ceroid accumulation is that the pigment, including
177 MDA adducts, is autofluorescent (20). We identified prominent autofluorescent F4/80⁺
178 aggregates in WT mice which were greatly reduced in stabilin-1^{-/-} mice in both CCl₄- and
179 MCD diet-induced liver injury (Fig.2G,H). We then showed that F4/80⁺ ceroid-laden
180 macrophages in experimental liver injury in WT mice co-express stabilin-1 (Fig.2I).
181 We used a thiobarbituric acid reactive substances (TBARS) assay to detect serum MDA
182 and found similar levels in WT and stabilin-1^{-/-} mice after CCl₄ injury and MCD diet

183 suggesting that production of MDA is not reduced in stabilin-1 deficiency (Fig.2J).
184 Proteomic analysis of baseline serum samples demonstrated increased levels of several
185 proteins in the serum of stabilin-1^{-/-} mice compared to WT mice (SI Appendix Table S1)
186 several of which are related to oxidative stress. However we did not detect increased
187 circulatory levels of pro-inflammatory or pro-fibrogenic cytokines such as TGFβ, PDGF A
188 and B, in keeping with previous studies (13).
189 Our findings prompted us to study the distribution of ceroid-laden macrophages in relation
190 to sites and stage of fibrosis. We detected ceroid-laden macrophages along the hepatic
191 scar in WT mice (Fig.3A) whereas in stabilin-1 deficient animals, the absence of ceroid
192 macrophages was associated with a dense network of collagen III in the hepatic scar
193 (Fig.3B). Further analysis of livers after 4 weeks resolution demonstrated the persistence
194 of ceroid-laden macrophages in close proximity to residual hepatic scar in WT mice
195 (Fig.3C). In contrast, ceroid-laden macrophages were not seen in stabilin-1^{-/-} mice in which
196 extensive collagen-rich scars persisted after 4 weeks of resolution (Fig.3D). Thus,
197 collectively our data suggest that stabilin-1 on macrophages protects against liver fibrosis
198 by taking up fibrogenic modified lipids.

199

200 **Stabilin-1 deficiency is associated with excess CCL3 production in liver tissue and**
201 **stabilin-1 suppresses CCL3 expression in macrophages.**

202 To identify specific mediators of fibrogenesis in stabilin-1 deficiency, we used RNA-seq to
203 compare the gene expression profile of liver tissue from WT and stabilin-1^{-/-} mice. A
204 number of genes showed increased expression in stabilin-1^{-/-} mice (SI Appendix Table S2)
205 but the only established fibrogenic mediator which was increased was the chemokine CCL3
206 (21, 22). We validated our findings with qPCR of liver tissue and critically demonstrated
207 that CCL3 was persistently elevated after four weeks of resolution in stabilin-1^{-/-} mice

(Fig.4A). In support of these findings we detected increased CCL3 protein in stabilin-1^{-/-} mice which colocalised with hepatic macrophages (Fig. 4B-D). We investigated whether the pro-fibrogenic phenotype in livers of stabilin-1^{-/-} mice was linked to ox-LDL uptake to form ceroid-laden macrophages. We initially confirmed the presence of autofluorescent stabilin-1⁺ ceroid-laden macrophages in livers from patients with chronic liver diseases (SI Appendix Fig. S6A,B). As MDA-LDL is one of the most abundant products of lipid peroxidation and contributes to ceroid formation (23), we investigated whether stabilin-1 on human macrophages is involved in its uptake and degradation. We isolated monocytes from human peripheral blood and stimulated their stabilin-1 expression *in vitro* (24). After 2 hours of MDA-LDL incubation we were able to detect intracellular deposits of MDA-LDL completely co-localised with stabilin-1 (SI Appendix Fig. S7A). Uptake of MDA-LDL was minimal in unstimulated monocytes which had low expression of stabilin-1 (SI Appendix Fig. S7B). A function blocking antibody to stabilin-1 led to the internalisation of stabilin-1 thereby inhibiting MDA-LDL uptake (Fig. 5A,B). We also linked the scavenging function of stabilin-1 positive macrophages to CCL3 expression by demonstrating a significant reduction in macrophage CCL3 transcription after MDA-LDL uptake compared to control (Fig. 5C). We could reverse this effect using the function blocking antibody against stabilin-1 (Fig. 5D). These results suggest that stabilin-1 expression is critical for the uptake of MDA-LDL by macrophages, a process which also modulates macrophage-derived CCL3 secretion.

228

Stabilin-1 deficiency is associated with a pro-inflammatory hepatic macrophage phenotype.

To assess how stabilin-1 deficiency influenced macrophage phenotype we sorted macrophages from CCl₄ treated livers. Analysis of WT hepatic macrophage populations

233 during liver injury demonstrated higher transcript expression of stabilin-1 in F4/80^{hi}/CD11b^{lo}
234 compared to the F4/80^{lo}/CD11b^{hi} population (SI Appendix Fig S8A). The loss of stabilin-1
235 led to higher expression of M1 markers CCL3 and TNF α in both F4/80^{hi}/CD11b^{lo} and F4/80
236 ^{lo}/CD11b^{hi} subsets. M2 marker MMP-9 was also elevated but Arginase-1 was reduced in
237 the F4/80^{hi}/CD11b^{lo} subset (SI Appendix Fig. S8B-E). This is in keeping with previous
238 studies where defining macrophages through their polarisation to classical M1 or M2
239 macrophages is unreliable in liver injury as M1/M2 markers can be expressed
240 simultaneously by liver macrophages (25).
241 Ramachandran *et al.* demonstrated that the CD11b^{hi}Ly6C^{lo} monocyte-derived macrophage
242 population increases significantly during fibrosis resolution and functions as the 'restorative'
243 macrophage (25) leading us to compare populations of 'pro-fibrotic' CD11b^{hi}Ly6C^{hi} and
244 'restorative' CD11b^{hi} Ly6C^{lo} macrophages. Liver injury in both WT and stabilin-1^{-/-} mice
245 resulted in a predominance of Ly6C^{hi} macrophages in the liver (SI Appendix Fig. S8F,G).
246 During resolution there was a shift towards Ly6C^{lo} macrophages in the WT group which
247 was not seen in the stabilin-1^{-/-} mice (SI Appendix Fig.S8H). Intrahepatic lymphocyte
248 populations did not differ between WT and stabilin-1^{-/-} mice after the injury or during
249 resolution (SI Appendix Fig. S9B-K). Collectively, these results demonstrate that the
250 profibrogenic response in stabilin-1 deficiency is associated with a pro-inflammatory
251 macrophage phenotype during injury.

252

253 **Deletion of stabilin-1 in myeloid cells is associated with a loss of ceroid-laden**
254 **macrophages and exacerbated fibrosis.**

255 The preceding results suggest that stabilin-1 protects the liver from fibrosis by allowing
256 macrophages to take up and remove pro-fibrogenic lipid peroxidation products and at the
257 same time suppressing CCL3 production. To test this hypothesis *in vivo* we used Tie-2 Cre

258 and Lys2 Cre strains to generate cell-selective knockouts. We have previously confirmed
259 their selectivity and efficiency in knocking down stabilin-1 (26). We have shown that in our
260 Tie-2 Cre model stabilin-1 is absent from endothelium (ENDO stab-1^{-/-}) but macrophage
261 expression is maintained. In our Lys2 Cre model stabilin-1 is absent from the myeloid
262 population (MACRO stab-1^{-/-}). In practice this model is selective for macrophages, since
263 neutrophils do not express stabilin-1 and therefore are unaffected by this knockout. We
264 confirmed this specificity in the livers of our cell selective strains at baseline and during liver
265 injury (SI Appendix Fig. S10A,B).

266 Fibrosis in ENDO stab-1^{-/-} mice was comparable to WT mice in both CCl₄ liver injury and
267 MCD diet (SI Appendix Fig. S11A-C), whereas fibrogenesis was increased in MACRO stab-
268 1^{-/-} animals (Fig.6A,B) with significantly more accumulation of collagen III associated with
269 increased αSMA staining in MACRO stab-1^{-/-} mice versus WT after CCl₄ injury (Fig.6C-F).
270 Fibrosis resolution was delayed in the MACRO stab-1^{-/-} animals as demonstrated by
271 persistently elevated transcript levels of αSMA and increased hydroxyproline staining and
272 critically for our hypothesis we found persistent elevation of CCL3 compared to WT animals
273 (Fig.6G-I) and increased serum ALT levels in response to CCl₄ as seen in the full knockout
274 (Fig.6J). We also detected significantly elevated hydroxyproline content in MACRO stab-1^{-/-}
275 mice compared to WT mice after MCD diet (SI Appendix Fig.S11D). Thus the increased
276 fibrosis in response to liver injury seen in the absence of stabilin-1 is predominantly
277 mediated through stabilin-1 expressing macrophages.

278 There were no detectable differences in the numbers of ceroid-laden macrophages in the
279 livers of WT and ENDO stab-1^{-/-} mice (SI Appendix Fig. S11E,F). In contrast, very few
280 ceroid-laden macrophages were seen in the livers of CCl₄ treated MACRO stab-1^{-/-} animals
281 (SI Appendix Fig. S11E,F). To further confirm the role of macrophage stabilin-1 in fibrosis
282 resolution we undertook experiments similar in design to those described by

283 Ramachandran *et al.* (25). This consisted of a 4 week model of CCl₄ liver injury performed
284 in MACRO stab-1^{-/-} and WT mice followed by adoptive transfer of wild type myeloid cell
285 elements or vehicle control at 24 and 72 hours after the final injection of CCl₄ followed by
286 an analysis of fibrogenesis at 120 hours. Transcription of fibrogenic markers and collagen
287 III expression in liver tissue showed no differences between WT mice receiving myeloid
288 cells or vehicle without cells (SI Appendix Fig S12A,D,F), whereas in the MACRO stab-1^{-/-}
289 there was a trend of reduced transcription in nearly all fibrogenic markers in mice receiving
290 wild type myeloid cells (SI Appendix Fig.S12B). We confirmed the presence of adoptively
291 transferred Dsred⁺ myeloid cells within liver tissue at sites of collagen deposition (SI
292 Appendix Fig. S12C). Finally, we demonstrated a significant reduction in hepatic collagen
293 III expression in MACRO stabilin-1^{-/-} mice receiving wild type myeloid cells compared to
294 vehicle control (SI Appendix Fig. 12E,F).

295

296 **Discussion**

297 This study reports a novel mechanism which involves a subset of stabilin-1⁺ macrophages
298 found in both experimental and human liver injury which play a critical role in protecting
299 against excessive fibrosis in response to oxidative stress. Stabilin-1 deficiency led to an
300 increase in hepatic CCL3 associated with the recruitment of GFAP⁺ fibroblasts and an
301 increase in baseline hepatic fibrosis. Using cell-specific knockout animals we were able to
302 show that stabilin-1 mediates its effects by enabling macrophages to take up and clear
303 fibrogenic oxidised lipids generated in response to liver injury. Stabilin-1 deficiency was
304 associated with a marked reduction of ceroid-laden macrophages during liver injury. Ceroid-
305 laden macrophages are a well recognised pathological feature of liver injury but, to our
306 knowledge, their contribution to liver fibrosis is unknown. Ceroid contains modified
307 lipoproteins (such as MDA-LDL) which are generated as a consequence of chronic

oxidative stress. We found in both humans and mice that they are derived from a subset of macrophages, that upregulate stabilin-1. The expression of stabilin-1 allows macrophages to take up and clear modified LDLs and in addition we show that this uptake suppresses the secretion of the pro-fibrogenic chemokine CCL3 resulting in reduced fibrosis and the promotion of scar resolution. The highest levels of stabilin-1 were detected in the F4/80^{hi} CD11b^{lo} population suggesting that tissue resident macrophages may upregulate stabilin-1 during liver injury. Interestingly, stabilin-1 deficiency was associated with an inflammatory phenotype in both infiltrating monocytes and mature tissue resident macrophages. The therapeutic potential of stabilin-1⁺ macrophages was demonstrated by our finding that the adoptive transfer of wild type myeloid cells can promote resolution of fibrosis in stabilin-1 deficiency. These results suggest that macrophages which are stimulated or engineered to express high levels of stabilin-1 could be a potential cell therapy in fibrotic liver disease. In addition to its potential role in liver disease we suggest that stabilin-1 plays a role in tissue homeostasis by removing local products of low level oxidative stress. This explains why increased fibrosis is seen in stabilin-1 deficient mice even in the absence of exogenous injury. The liver is constantly exposed to bacterial products and xenobiotics from both the portal and systemic circulation and under normal conditions stabilin-1 on endothelium as well as on macrophages may allow the rapid removal of products of oxidative stress thereby preventing low level continuous injury and scarring. Whereas in response to liver injury, protection and resolution requires the involvement of stabilin-1⁺ macrophages. These findings describe a novel mechanism involved in the regulation of tissue fibrosis that allows efficient wound healing without destructive scarring in response to liver injury.

330

331 **Methods**

332

333 **Animals**

334 Stabilin-1 knockout mice and cell-specific mice were generated as previously described
335 (26). CAG-Dsred*MST^{1Nagy/J} (stock 005441) were from the Jackson Laboratory. All animal
336 studies were done in adherence with the rules and regulations of The Finnish Act on Animal
337 Experimentation (62/2006), and accepted by the local Committee for Animal
338 Experimentation (Animal licence number 5587/04.10.07/2014).

339 **Liver injury models**

340

341 *Carbon tetrachloride (CCl₄) injury model*

342 8-week-old mice were injected twice weekly for 8 weeks with either CCl₄ (1.0 ml/kg CCl₄
343 diluted 1:3 in mineral oil, Sigma-Aldrich) or a mineral oil vehicle control. Animals were
344 sacrificed 72 h after the final dose of CCl₄ (27) or after a 4-week recovery period.

345

346 Methionine Choline Deficient (MCD) Diet

347 Mice were fed an MCD diet (Harlan laboratories TD90262) for 6 weeks *ad libitum*. Control
348 animals received normal chow for 6 weeks (27).

349

350 **Human tissue**

351 Tissue and blood samples from patients were obtained with written informed consent and
352 with local ethics committee approval (LREC reference 06/Q2702/61 South Birmingham,
353 Birmingham, UK and 04/Q2708/41 South Birmingham, Birmingham, UK).

354

355 Statistical analysis , see SI Appendix Supplementary methods.

356

357 **Acknowledgements**

358 We are grateful to our clinical colleagues and patient donors for the provision of blood and
359 tissue samples. SS was funded by a Wellcome Trust Intermediate Clinical Fellowship

360 (097162/Z/11/Z). This work was funded by the Finnish Academy and the Sigrid Juselius
361 Foundation.

362 This paper presents independent research funded by the National Institute for Health
363 Research (NIHR). The views expressed are those of the authors and not necessarily those
364 of the NHS, the NIHR or the Department of Health.

365

366

367 **References**

368

369

370

- 371 1. Kocabayoglu P & Friedman SL (2013) Cellular basis of hepatic fibrosis and its role in
372 inflammation and cancer. *Front Biosci (Schol Ed)* 5:217-230.
- 373 2. Schneiderhan W, *et al.* (2001) Oxidized low-density lipoproteins bind to the scavenger
374 receptor, CD36, of hepatic stellate cells and stimulate extracellular matrix synthesis.
375 *Hepatology* 34(4 Pt 1):729-737.
- 376 3. Kang Q & Chen A (2009) Curcumin suppresses expression of low-density lipoprotein
377 (LDL) receptor, leading to the inhibition of LDL-induced activation of hepatic stellate
378 cells. *Br J Pharmacol* 157(8):1354-1367.
- 379 4. Xie G, *et al.* (2012) Role of differentiation of liver sinusoidal endothelial cells in
380 progression and regression of hepatic fibrosis in rats. *Gastroenterology* 142(4):918-927
381 e916.
- 382 5. Duffield JS, *et al.* (2005) Selective depletion of macrophages reveals distinct, opposing
383 roles during liver injury and repair. *J Clin Invest* 115(1):56-65.
- 384 6. Kzhyshkowska J (2010) Multifunctional receptor stabilin-1 in homeostasis and disease.
385 *ScientificWorldJournal* 10:2039-2053.
- 386 7. Li R, *et al.* (2011) Role of liver sinusoidal endothelial cells and stabilins in elimination of
387 oxidized low-density lipoproteins. *Am J Physiol Gastrointest Liver Physiol* 300(1):G71-81.
- 388 8. Workman G & Sage EH (2011) Identification of a sequence in the matricellular protein
389 SPARC that interacts with the scavenger receptor stabilin-1. *J Cell Biochem* 112(4):1003-
390 1008.
- 391 9. Kzhyshkowska J, *et al.* (2008) Alternatively activated macrophages regulate
392 extracellular levels of the hormone placental lactogen via receptor-mediated uptake and
393 transcytosis. *J Immunol* 180(5):3028-3037.
- 394 10. Shetty S, *et al.* (2011) Common lymphatic endothelial and vascular endothelial receptor-
395 1 mediates the transmigration of regulatory T cells across human hepatic sinusoidal
396 endothelium. *J Immunol* 186(7):4147-4155.
- 397 11. Iredale JP (2007) Models of liver fibrosis: exploring the dynamic nature of inflammation
398 and repair in a solid organ. *J Clin Invest* 117(3):539-548.
- 399 12. Varela-Rey M, *et al.* (2009) Non-alcoholic steatohepatitis and animal models:
400 understanding the human disease. *Int J Biochem Cell Biol* 41(5):969-976.

13. Schledzewski K, *et al.* (2011) Deficiency of liver sinusoidal scavenger receptors stabilin-1 and -2 in mice causes glomerulofibrotic nephropathy via impaired hepatic clearance of noxious blood factors. *J Clin Invest* 121(2):703-714.
14. Benyon RC, *et al.* (1999) Progelatinase A is produced and activated by rat hepatic stellate cells and promotes their proliferation. *Hepatology* 30(4):977-986.
15. Cassiman D, Libbrecht L, Desmet V, Denef C, & Roskams T (2002) Hepatic stellate cell/myofibroblast subpopulations in fibrotic human and rat livers. *J Hepatol* 36(2):200-209.
16. Wynn TA & Barron L (2010) Macrophages: master regulators of inflammation and fibrosis. *Semin Liver Dis* 30(3):245-257.
17. Fallowfield JA, *et al.* (2007) Scar-associated macrophages are a major source of hepatic matrix metalloproteinase-13 and facilitate the resolution of murine hepatic fibrosis. *J Immunol* 178(8):5288-5295.
18. Salguero Palacios R, *et al.* (2008) Activation of hepatic stellate cells is associated with cytokine expression in thioacetamide-induced hepatic fibrosis in mice. *Lab Invest* 88(11):1192-1203.
19. Yamada S, *et al.* (2001) Immunochemical detection of a lipofuscin-like fluorophore derived from malondialdehyde and lysine. *J Lipid Res* 42(8):1187-1196.
20. Seehafer SS & Pearce DA (2006) You say lipofuscin, we say ceroid: defining autofluorescent storage material. *Neurobiol Aging* 27(4):576-588.
21. Heinrichs D, *et al.* (2013) The chemokine CCL3 promotes experimental liver fibrosis in mice. *PLoS One* 8(6):e66106.
22. Seki E, *et al.* (2009) CCR1 and CCR5 promote hepatic fibrosis in mice. *J Clin Invest* 119(7):1858-1870.
23. Esterbauer H, Gebicki J, Puhl H, & Jurgens G (1992) The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic Biol Med* 13(4):341-390.
24. Politz O, *et al.* (2002) Stabilin-1 and -2 constitute a novel family of fasciclin-like hyaluronan receptor homologues. *Biochem J* 362(Pt 1):155-164.
25. Ramachandran P, *et al.* (2012) Differential Ly-6C expression identifies the recruited macrophage phenotype, which orchestrates the regression of murine liver fibrosis. *Proc Natl Acad Sci U S A* 109(46):E3186-3195.
26. Karikoski M, *et al.* (2014) Clever-1/stabilin-1 controls cancer growth and metastasis. *Clin Cancer Res* 20(24):6452-6464.
27. Weston CJ, *et al.* (2015) Vascular adhesion protein-1 promotes liver inflammation and drives hepatic fibrosis. *J Clin Invest* 125(2):501-520.

Figure Legends

Figure 1

Stabilin-1 deficiency exacerbates fibrosis after CCl₄ liver injury. Wild-type (WT) and stabilin-1-deficient (stabilin-1^{-/-}) mice were subjected to CCl₄-induced liver injury: control (Oil); 8 week CCl₄ injury (CCl₄) and 4 week resolution after the CCl₄ injury (Res). (A,B) Sirius red stainings in livers (n=4-5 in each group). (C-H) Collagen I, Collagen III

immunostainings stainings and GFAP/ α SMA co-immunostainings and quantifications of liver sections (n=3-5 in each group). (I) Sirius red staining from livers after resolution. (J) mRNA expression of Collagen I and Collagen III in livers. (K) Hydroxyproline determinations in liver samples and (L) serum alanine transaminase (ALT) levels. (for J-L n=5-6 mice in each group). Statistical significance was determined by unpaired *t*-test (G,H,J)) and 1-way ANOVA analysis, with a Tukey's *post-hoc* multiple comparison test (K, L). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0005$. Bars 200 μ m (A-D, I) Bars 50 μ m (E, F)

Figure 2

Stabilin-1 deficiency is associated with a reduction of ceroid-laden macrophages during liver injury. (A,B) Staining of livers for F4/80 from WT and stabilin-1^{-/-} mice in oil controls and CCl₄ injury. Arrows indicate aggregates of F4/80⁺ cells. (C) Quantification of F4/80 positive area staining in WT and stabilin-1^{-/-} mice in oil controls and CCl₄ injury. (D) Periodic acid schiff-diastase (PAS-D) staining of livers from WT and stabilin-1^{-/-} mice after CCl₄ injury (black arrows highlight PAS-D positive cells and white arrows highlight areas of increased scar formation. (E) Staining of livers for Malondialdehyde (MDA) from WT and stabilin-1^{-/-} mice after CCl₄ injury (F) Quantification of MDA positive area staining in WT and stabilin-1^{-/-} mice after CCl₄ injury. (n=3-4 mice in each group) Representative high magnification fields in black box areas. (G) Autofluorescent ceroid aggregates (red) co-stained with F4/80 (green) in WT and stabilin-1^{-/-} mice after CCl₄ injury. (H) Quantification of ceroid staining within F4/80⁺ cells in livers from WT and stabilin-1^{-/-} mice after CCl₄ injury and MCD diet. (n=5 mice in each group). (I) Immunofluorescent staining of livers from WT mice after CCl₄ injury double stained for F4/80 (green), stabilin-1 (orange) and autofluorescent ceroid (red). (J) Measurement of serum MDA levels using TBARS assay in WT and stabilin-1^{-/-} mice after CCl₄ injury and MCD diet-induced injury (n=4 mice in each

group). Statistical significance was determined by unpaired *t*-test (C,F,H,J) * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$. Bars 100 μm (A,B), 200 μm (D,E), 50 μm (G), 10 μm (I).

474

475 **Figure 3**

476 **Loss of ceroid-laden macrophages is associated with exacerbation of hepatic**
477 **scarring.**

478 (A,B) Immunofluorescent staining of WT and stabilin-1^{-/-} mice after CCl₄ injury and (C,D) 4
479 weeks resolution for F4/80⁺ cells (green) and Collagen III (red). Right hand panels are
480 magnification of inset boxes in left hand panels (arrows highlight ceroid-laden
481 macrophages). Bars 200 μm (A-D).

482

483

484 **Figure 4**

485 **Stabilin-1 deficiency is associated with increased intrahepatic CCL3.** Wild-type (WT)
486 and stabilin-1-deficient (stab-1^{-/-}) mice were subjected to CCl₄-induced liver injury: control
487 (Oil); 8 week CCl₄ injury (CCl₄) and 4 week resolution after the CCl₄ injury (Res). (A) mRNA
488 expression of CCL3 in livers (n=5-7 mice in each group). (B) Immunofluorescent staining of
489 WT and stabilin-1^{-/-} mice in control (Oil) livers of F4/80⁺ cells (red) and CCL3 (green) and
490 (C) quantification of CCL3 staining (n=3 in each group for B,C). (D) High magnification
491 image of co-staining for F4/80⁺ cells (red) and CCL3 (green) from stabilin-1-deficient
492 (stabilin-1^{-/-}) liver. Statistical significance was determined by unpaired *t*-test (A,C) * $P < 0.05$,
493 ** $P < 0.005$, Bars 50 μm (B), 5 μm (D).

494

495 **Figure 5**

496 **Stabilin-1 suppresses CCL3 expression during Malondialdehyde-LDL uptake by**
497 **macrophages.**

498 (A) Immunofluorescent staining of IL-4/Dex cultured human monocytes pre-treated with
 499 isotype control and or (B) Stabilin-1 function blocking antibody (3-372) followed by
 500 incubation with MDA-LDL (10 µg/ml) for 2 h. Representative images from three separate
 501 cell isolates. (C) Comparison of mRNA expression of CCL3 in IL-4/Dex cultured human
 502 monocytes (control) and those exposed to MDA-LDL for 24h. (D) Comparison of mRNA
 503 expression of CCL3 in IL-4/Dex cultured human monocytes exposed to MDA-LDL for
 504 24hours, pre incubated with IgG1 antibody (control; 10 µg/ml) or 3-372 antibody (Stab-1
 505 blocking antibody; 10 µg/ml) (n=3 independent experiments). Statistical significance was
 506 determined by a paired t-test. *P<0.05, ***P<0.005. Bar 10 µm (A,B),

507

508 **Figure 6**

509

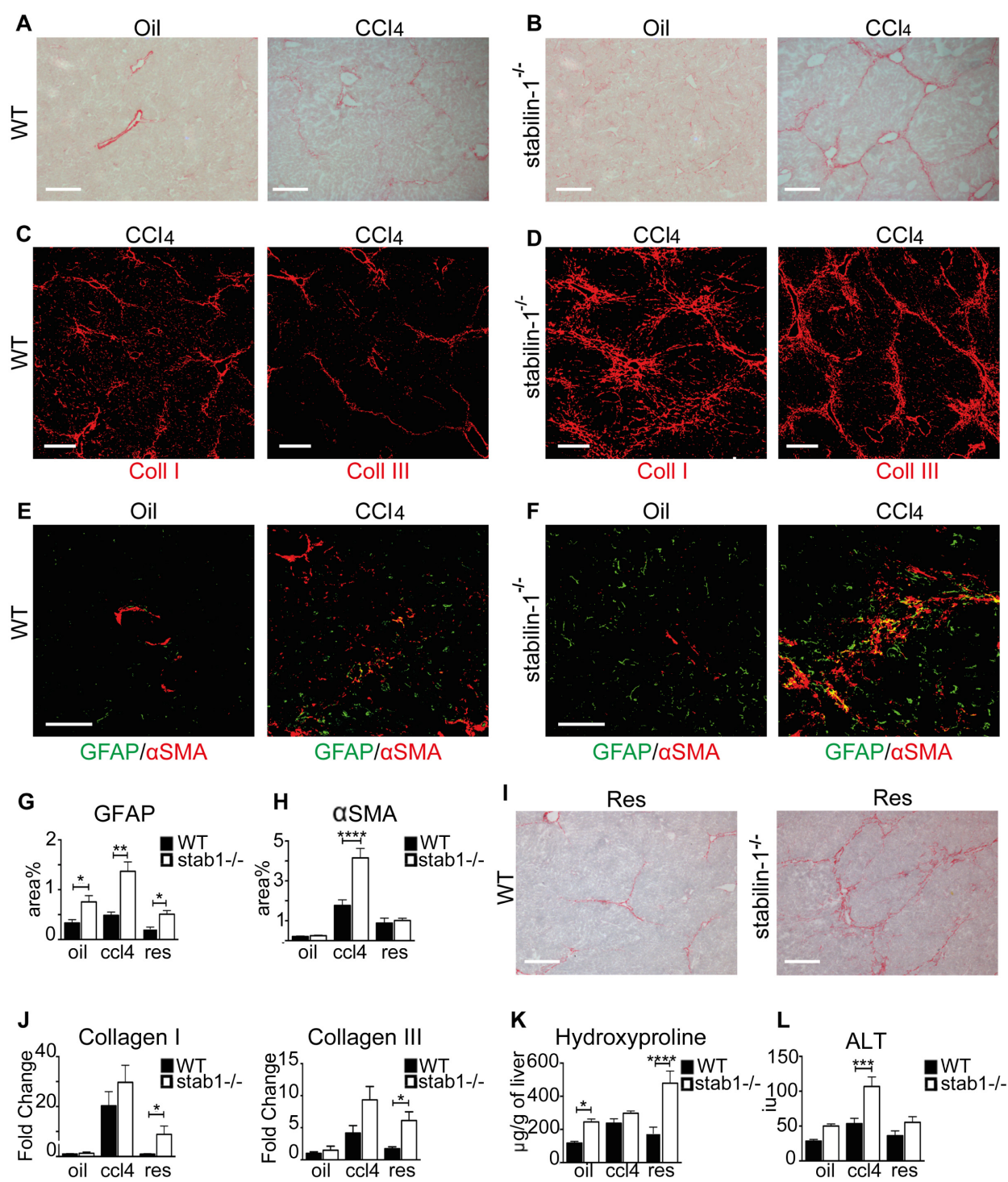
510 **Stabilin-1 deficiency on macrophages leads to increased ECM deposition.** Wild-type
 511 (WT) and macrophage stabilin-1-deficient (MACRO stab-1^{-/-}) mice were subjected to (A-H)
 512 CCl₄-induced liver injury: control (Oil); 8 week CCl₄ injury (CCl₄) and 4 week resolution after
 513 the CCl₄ injury (Res). (A,B) Sirius red stainings in livers. (C-F) Collagen I, Collagen III and
 514 αSMA immunostainings and quantifications of liver sections. (G) mRNA expression of
 515 αSMA and CCL3 in livers, (for A-G n=4-6 mice in each group). (H) Hydroxyproline
 516 determinations in liver samples (n=5-6 mice in each group) and serum alanine
 517 transaminase (ALT) levels (n=4-5 mice in each group). Statistical significance was
 518 determined by unpaired *t*-test (E) and 1-way ANOVA analysis, with a Tukey's *post-hoc*
 519 multiple comparison test (G, H). * P<0.05, ***P<0.001. Bars 200 µm (A-D). Bars 50 µm
 520 (F).

521

522

523

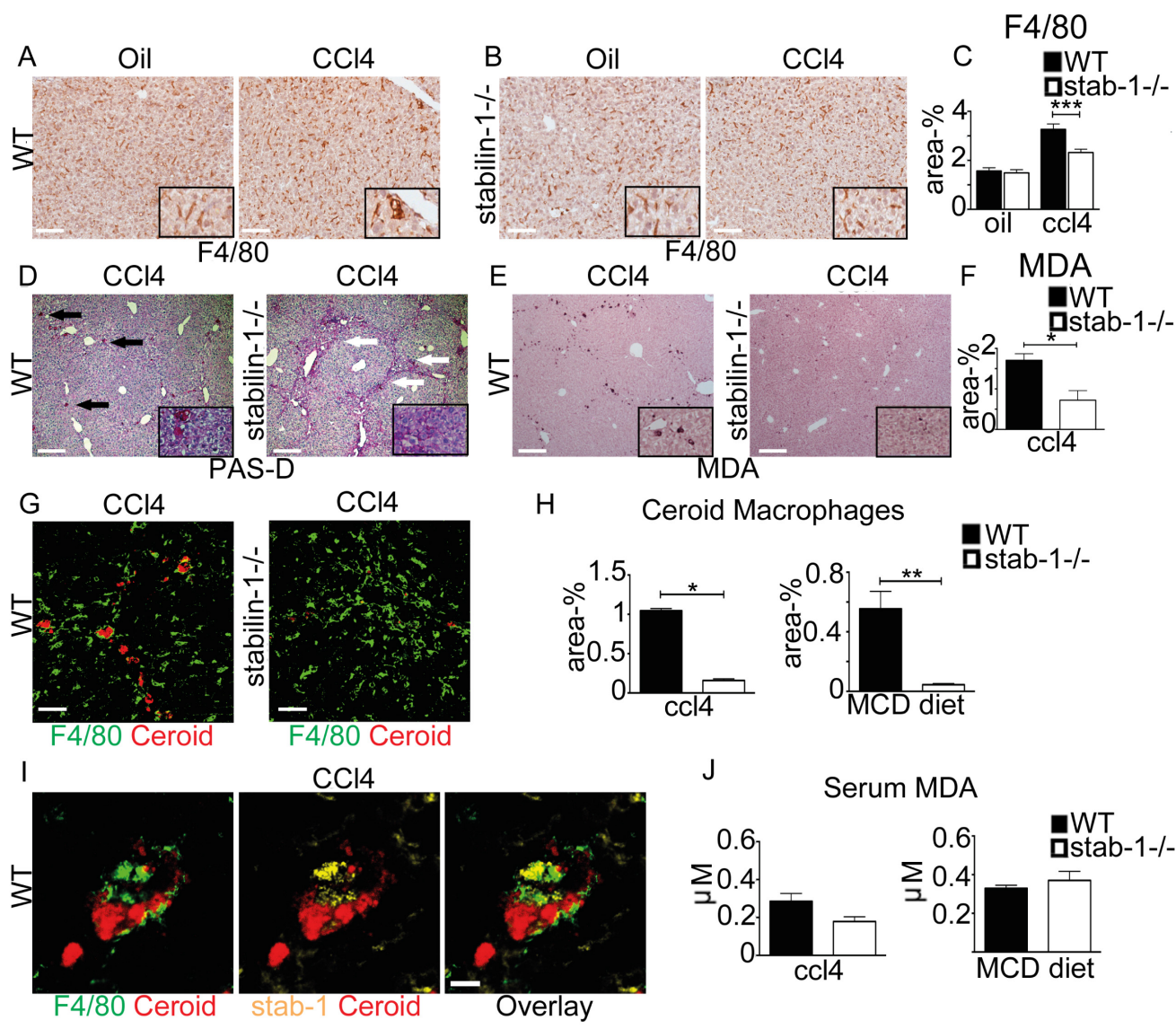
524 **Figure 1**



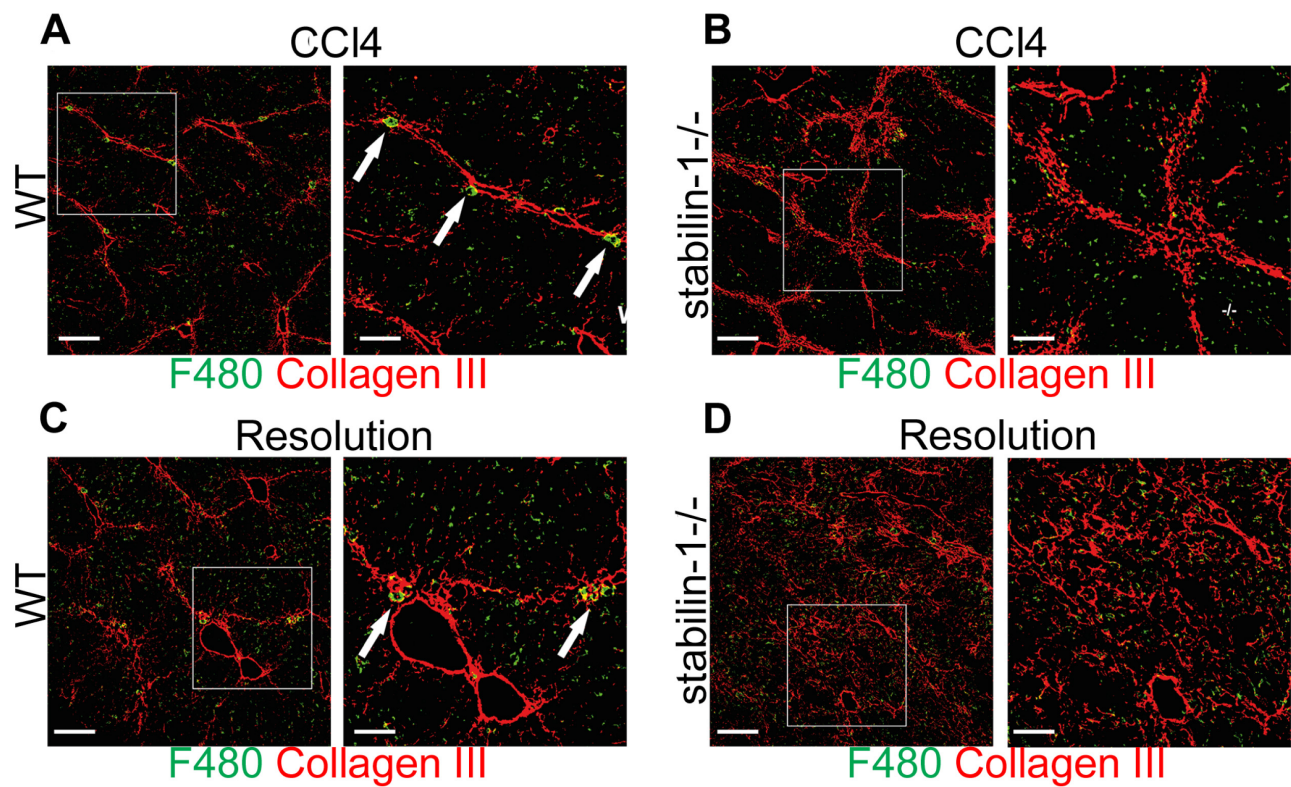
525

526

527 **Figure 2**



536 **Figure 3**



537

538

539

540

541

542

543

544

545

546

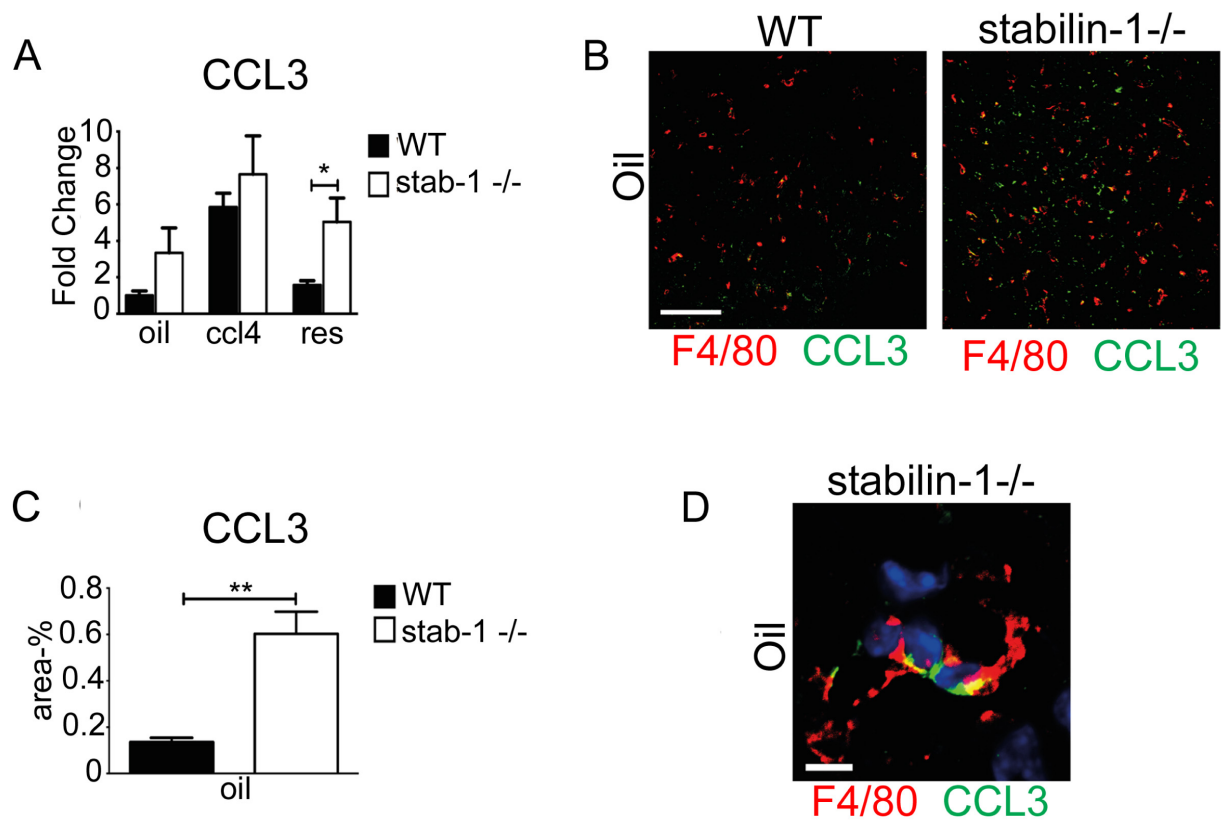
547

548

549

550

551 **Figure 4**



552

553

554

555

556

557

558

559

560

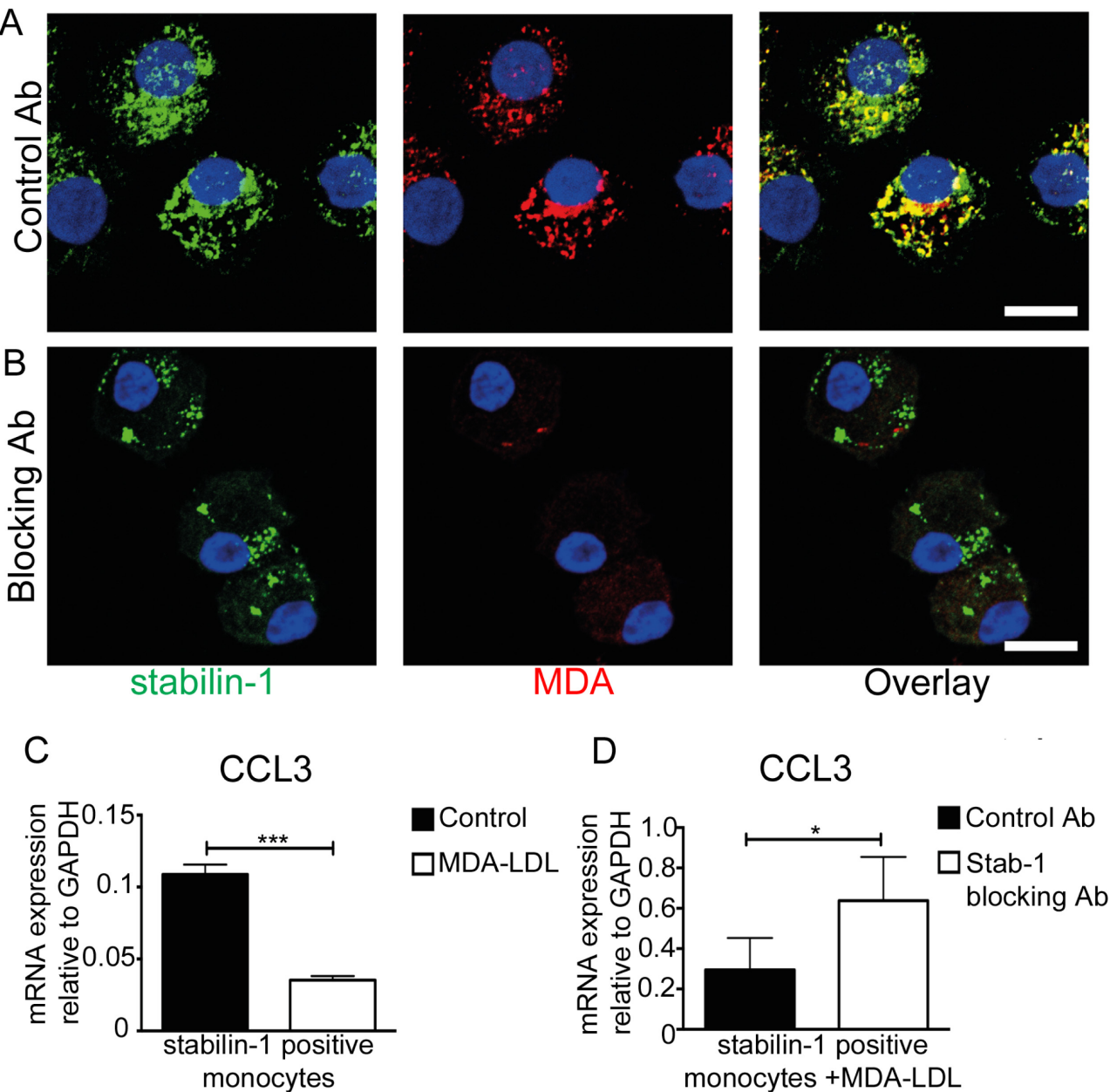
561

562

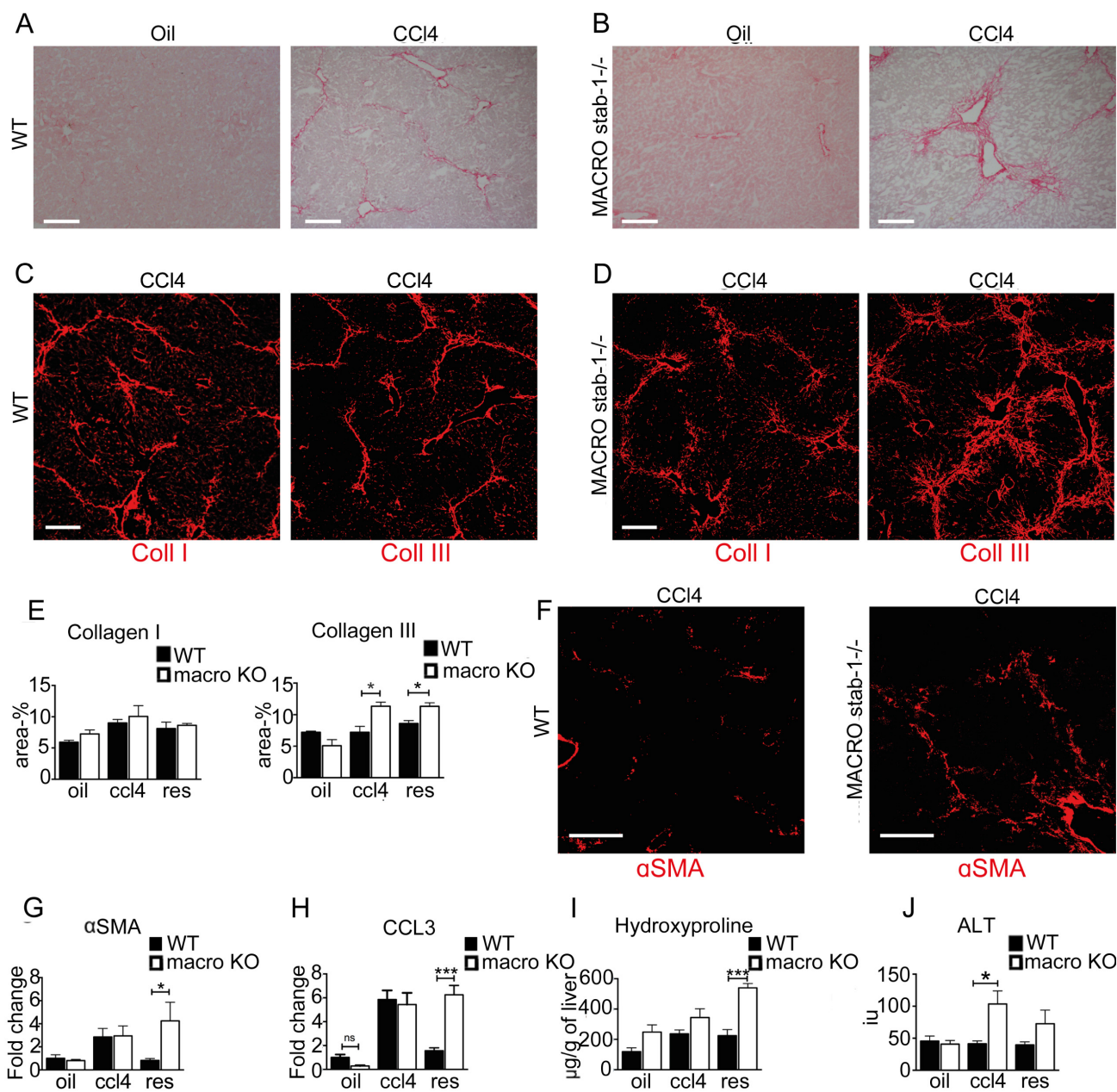
563

564

565 **Figure 5**



573 **Figure 6**



574